Characterization of Pemphigus Foliaceus Antigen from Human Epidermis

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Pemphigus foliaceus (PF) and its endemic form, Fogo Selvagem (FS), are characterized by subcorneal vesicles and pathogenic IgG autoantibodies directed against keratinocyte surface antigens. A major pool of FS antigen(s) remains bound to the insoluble epidermal envelope fraction. In this paper we demonstrate that this antigen(s) can be released from the envelope fraction by sonication. By immune precipitation four components can be detected, having molecular weights (MW) of 260, 80, 62, and 45 kD. The 260-kD component is lost by boiling or extraction with glycine HCl at pH 2.8. The major components appear to be the 80- and 62-kD poly-peptides. They chromatograph as a unit by gel filtration in 0.1% SDS, in the MW range of 115–120 kD. The FS antigen(s) appears to be cationic, forming insoluble complexes at low pH with SDS, and is labile to ammonium sulfate and freezing and thawing. It is unaffected by positive pressure concentration, 50% acetone precipitation, and reduction/alkylation. The FS antigen(s) is precipitated by all FS and nonendemic PF sera except those in complete clinical and serologic remission. The FS antigen(s) is also precipitated by 50% of pemphigus vulgaris but none of the bullous pemphigoid sera tested. All FS antigenic components are immunoprecipitated by IgG4 autoantibodies, but the IgG1 subclass from the same patients appear to immunoprecipitate only the 62-kD polypeptide. The FS antigen(s) is able to adsorb human autoantibodies against human desmoglein 1 (DG1), but not rabbit antisera against bovine DG1 or 2. This paper shows that physical stress, i.e., sonication, may be able to solubilize sufficient FS antigen(s) from the epidermal envelope fractions for further chemical characterization. The relationship of these FS antigen(s) to other reported FS antigens is presently unknown. *J Invest Dermatol 96:815–821, 1991*

Endemic pemphigus foliaceus (PF) or Fogo Selvagem (FS) and nonendemic PF are autoimmune blistering diseases characterized by subcorneal vesicles and IgG class autoantibodies, which by indirect immunofluorescence (IF) stain the epidermal intercellular spaces (ICS) [1]. These autoantibodies are restricted predominantly to the IgG4 subclass and they are pathogenic in vivo, as shown by passive transfer experiments [2].

The precise molecular characterization of the antigenic moieties recognized by PF autoantibodies remains obscure. Stanley and colleagues were the first to show by immunoblotting procedures that one-third of the sera of FS and nonendemic PF patients bind desmoglein 1 (DG1) from epidermal extracts [3,4]. Because DG1 is a transmembrane glycoprotein [5,6] and FS autoantibodies bind the surface of keratinocytes [7], it is thought that these autoantibodies recognize the extracellular domain of this molecule.

In previous publications we have reported the identification of two FS antigens from human epidermis, one obtained by digestion of epidermal envelope fractions with papain [8] and the other by trypsinization of viable skin [9]. The molecular weight (MW) of these FS antigens were of 50 kD and 45 kD, respectively, and were recognized by sera of patients with endemic and nonendemic PF, as well as by approximately one-half of the sera of patients with pemphigus vulgaris (PV).

The major drawback of these procedures is that the quantity of antigen obtained is insufficient for further chemical characterization. In this report we describe methods to increase the yield and further characterize the FS antigen(s). The antigen(s) immunoprecipitated by FS sera exhibit a 260-MW complex in 5% SDS-PAGE using reduced, unboiled samples. This complex becomes undetectable upon boiling or by lowering the pH to 2.8 with glycine HCl. Under these conditions three lower-MW bands become prominent, two strong ones of 80 kD and 62 kD and a third weak one of 45 kD. The 80-kD and 62-kD bands chromatographed as a single peak after IgG on Bio Gel A-1.5m chromatography. This approach will facilitate the biochemical analysis of these epidermal antigens.

Manuscript received October 5, 1990; accepted for publication February 21, 1991.

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Supported in part by U.S. Public Health Service Grants RO1-AR32599, RO1-AR32081, from the National Institutes of Health. Dr. Ciro R. Martins is a Post Doctoral Fellow supported by a grant from Merrell Dow Pharmaceuticals awarded by the Dermatology Foundation.

Abbreviations:
BP: bullous pemphigoid
DG1: desmoglein 1
DG2: desmoglein 2
FS: fogo selvagem
ICS: intercellular spaces
IF: immunofluorescence
IPB: immunoprecipitation buffer
NHS: normal human serum
PAGE: polyacrylamide gel electrophoresis
PF: pemphigus foliaceus
PMSF: phenylmethylsulfonylfluoride
PV: pemphigus vulgaris
SaC: staphylococcus aureus cells
SDS: sodium dodecyl sulfate
SPA: staphylococcus aureus protein A
TBS: tris-buffered saline

0022-202X/91/$03.50

MATERIALS AND METHODS

Sources of Sera FS sera were obtained from patients with active and widespread disease showing high titers of FS autoantibodies (n:12). Ten FS patients in either spontaneous or steroid-induced complete clinical remission, possessing no FS autoantibodies (n:7) or low titers [1:40 (n:1), 1:160 (n:2)], were also included. Additionally, sera from PV patients (n:11), nonendemic PF (n:6), and bullous pemphigoid (BP) (n:11) were also tested. Control normal humn sera (NHS) were obtained from unaffected individuals living in endemic areas of FS in Brazil (n:6) and from normal laboratory volunteers (n:6).

A polyclonal rabbit antiserum against bovine DG1 (B-38) supplied by Dr. M. S. Steinberg (Princeton University) and a rabbit antiserum against bovine DG2 supplied by Dr. A. Kusumi (University of Tokyo) were also tested.

Murine monolonal antibodies against human IgG subclasses were obtained from two sources. For determinations of the IgG subclass of pemphigus autoantibodies by indirect immunofluorescence (IF) we used monoclonal antibodies kindly supplied by Dr. Theofilopoulus (Scripps Clinic, La Jolla, CA). The specificity of these autoantibodies have been reported elsewhere [2]. For immunoprecipitation we used monoclonal antibodies prepared from the following hybridoma cell lines: HP6025, specific for human IgG4 and HP6058, specific for human IgG1, IgG2, and IgG3 (anti IgG1, 2, 3) obtained from the American Type Culture Collection. Monoclonal antibodies were partially purified from ascites fluid by gel filtration chromatography on Sephadex G-200 and were linked to CNBr-activated agarose [10].

Immunofluorescence Procedures All sera were screened for antiepidermal autoantibodies by routine indirect IF as described previously [2,10] using cryosections of human skin and fluorescein isothiocyanate (FITC)-conjugated goat antihuman IgG (Cappel Laboratories, Cochranville, PA). The IgG subclass of FS autoantibodies was determined by indirect IF procedures as reported previously [2] using monoclonal antibodies to individual human IgG subclasses. Specimens were analyzed using a Nikon fluorescence microscope equipped with epifluoresence.

Preparation of Human Insoluble Epidermal Fractions Containing FS Antigen(s) Skin obtained from surgical specimens from the plastic surgery service was stripped from the subcutaneous tissue. The epidermis was separated by heating at 56°C for 50 sec in a water bath, and then sequentially extracted as previously described [8]. Briefly, the epidermis was homogenized in 20 mM tris-buffered saline, pH 7.6, containing 5 mM $CaCl_2$ (TBS/Ca^{++}) and 1% Nonidet P-40 (NP-40) in the presence of a cocktail of protease inhibitors composed of 2 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mg/ml of each of the following reagents: peapstatin, antipain, chymostatin, and leupeptin (Sigma Chemical Co., St. Louis, MO). The insoluble pellet was subsequently extracted in a solution of 1.5 M KCl containing 5 mM $CaCl_2$. A suspension of the insoluble epidermal pellet was digested with trypsin (bovine pancreas, Sigma type III) at a concentration of 0.04 mg/ml in TBS/Ca^{++} for 2.0 h at room temperature with continuous shaking. Digestion was stopped by the addition of 2 mM PMSF. The trypsin-resistant pellet was separated by centrifugation at 15,000 × *g* for 25 min. The insoluble pellet was resuspended gently in cold TBS/Ca^{++} and treated with 2 mM PMSF and the cocktail of inhibitors described above. Trypsin media and TBS/Ca^{++} wash were stored at −20°C for further testing.

Preparation of Human Soluble Epidermal Fractions Containing FS Antigen(s) In preliminary studies we found that the trypsin-resistant epidermal pellets suspended in TBS/Ca^{++} released immunoreactive FS antigen by repeated cycles of sonication (30 sec, pulsed at 50% efficiency and 4 setting, [Sonicator W-385, Heat Systems-Ultransonics Inc., Farmingdale, NY]). After sonication the TBS/Ca^{++} soluble fraction was removed by centrifugation and tested for FS antigenic activity.

Preparation of 1% SDS Epidermal Extracts from Human Epidermis Human epidermis was extracted in 1% SDS in TBS without Ca^{++} using the same protease inhibitors listed above and following procedures described earlier [12]. This epidermal extract was run on 7.5% SDS-PAGE, transferred to nitrocellulose paper and used to test sera by immunoblotting techniques reported elsewhere [12].

Immunoadsorption of FS Autoantibodies by Epidermal Fractions Immunoadsorption of FS or PV autoantibodies by epidermal fractions was performed following previously reported procedures [8,9]. Briefly, varying amounts of the epidermal fraction [or bovine serum albumin (BSA) as a control] were incubated at room temperature for 1 h with an aliquot of a 1:80 dilution of a FS serum known to have an indirect IF titer of FS autoantibodies of 1:1280. Following centrifugation at 10,000 × *g* the supernatant was coded and tested by indirect IF for detection of pemphigus autoantibodies. To eliminate the possibility that a component of the unfractionated FS antigen was causing loss of the antigenicity of the cellular molecule in situ (epidermal cryosection), e.g., by proteolysis, the following experiment was carried out. Cryosections of human skin were placed on glass slides and incubated with the epidermal fraction for 30 min, followed by a rinse with TBS buffer. These sections were then used to test a positive FS serum by indirect IF.

The rabbit anti-DG1 was also adsorbed with the epidermal fraction and then tested against 1% SDS extracts of human epidermis by immunoblotting procedures.

The Stability of FS Antigen(s) Under a Variety of Experimental Conditions To investigate the stability of the FS antigen, aliquots of the epidermal fraction were subjected to a variety of experimental conditions including the following: boiling for 3 min, three cycles of freezing and thawing, lyophilization, concentration by ultrafiltration, overnight incubation at 37°C, and precipitation by 50% ammonium sulfate or 50% acetone at −20°C. Reduction with DTT and alkylation with iodocetamide, and changes in the pH, were also tested. Residual antigenic activity was tested in each sample by immunoadsorption of a positive FS serum and compared with an untreated epidermal fraction.

Labeling of FS Antigen(s) with ^{125}I Epidermal proteins were radiolabeled by the Chloramine T method [13]. Free iodine was removed by dialysis. The percentage of ^{125}I incorporated into the epidermal proteins and precipitable by 10% trichloroacetic acid was estimated. The final radiolabeled epidermal fraction was aliquoted and stored at −20°C for immunoprecipitation procedures.

Immunoprecipitation of ^{125}I-Labeled FS Antigen(s) by FS Sera Aliquots (0.1 ml) of radiolabeled FS antigen (10^7 cpm) in 0.4 ml 0.1 M tris/HCl buffer at pH 7.2, containing 5% BSA, 1% Triton X-100, and 5 mM $CaCl_2$ [immunoprecipitation buffer (IPB)] were incubated with 0.01 ml of patient or control serum for 1 h at room temperature. This was followed by incubation for 1 h with 0.05 ml of 10% dried Staphylococcus aureus cells (SaC) (Sigma, St. Louis, MO), suspension in IPB buffer, and centrifugation at 8,000 × *g* for 10 min. The pellet was washed three times with IPB buffer containing no BSA and then extracted in 100 μl of either 1% SDS or 0.2 M glycine HCl, pH 2.8. Similar procedures were followed when using agarose beads coated with monoclonal antibodies against human IgG subclasses. The samples were centrifuged and tested by SDS-PAGE under reduced and unreduced conditions without boiling. Separated proteins were analyzed by autoradiography.

Gel Electrophoresis and Autoradiography One-dimensional SDS-PAGE was performed as described previously [8,9] using 5% and 10% gels. The gel slabs were stained with Coomassie blue or silver stain and then dried for autoradiographic analysis. Dried gels were exposed to Kodak X-OMAT R (XR-5) film (Kodak, Rochester, NY) at −70°C for 24–48 h using intensifying screens. MW standards were used to calibrate the gels (Bio Rad, Richmond, CA).

Immunoblotting procedures were carried out following procedures described previously [12] using ^{125}I-labled *Staphylococcus*

aureus Protein A (SPA). Briefly, 1% SDS epidermal extracts separated by SDS-PAGE were transferred onto nitrocellulose paper and probed with test and control sera. Bound antibodies were demonstrated by autoradiography.

Fractionation of FS Antigen(s) by Gel Filtration Chromatography Radiolabeled FS antigens were precipitated by FS serum as described above and pooled. These antigens were solubilized in 0.1% SDS in TBS, pH 7.5, and applied to a 110 × 2.5-cm column containing Bio-Gel A 1.5m (Bio Rad, Richmond, CA) equilibrated with the same buffer. Fractions were monitored for radioactivity and optical density at 280 nm. Representative peaks were pooled and the molecular components precipitated with 50% acetone. The precipitates were redissolved in a small volume of TBS before SDS-PAGE and autoradiography.

RESULTS

Insoluble Epidermal Pellets Release FS Antigen(s) After Trypsinization Trypsin-resistant epidermal pellets released immunoreactive FS antigen by repeated sonication. This solubilized antigen was capable of blocking the staining of epidermal ICS produced by patient sera by indirect IF. Approximately 200 μg of total protein in the extract was able to eliminate completely the reactivity of 1 ml of a 1:80 dilution of FS sera (titer 1:1280). Protein concentration was estimated by absorbance of the antigen solution at 280 nm assuming an extinction of 1 mg/ml/OD unit. On gel electrophoresis, no bands could be detected by either Coomassie blue or silver stain. Direct IF examination of the residual pellets revealed substantial amounts of FS antigen still associated with this insoluble fraction.

Strikingly, insoluble epidermal pellets that had not been trypsinized did not release any antigenic material by sonication when tested in a similar fashion, nor did trypsin supernates that had not been sonicated. The antigenic activity in the soluble fraction was increased by concentration (Centricon p30), and was not abolished by overnight incubation at 37°C, by acetone precipitation, or by reduction/alkylation. The antigen was completely destroyed by boiling, repeated freeezing and thawing, 50% saturated ammonium sulfate, and lowering the pH to 2.5, followed by restoring it to pH 7.5. Lyophilization partially inactivated the antigenic activity.

Immunoprecipitation Studies The immunoprecipitated FS antigen(s) was run on 10% SDS PAGE (Fig 1). Autoradiography revealed three bands with MW of 80 kD, 62 kD, and 45 kD, precipitated by all sera from patients with active FS (Fig 1*A*) and all (n:6) nonendemic PF (not shown). The 80-kD and 62-kD bands were more intense than the 45-kD band, which varied in intensity from batch to batch and sometimes appeared as a doublet. It was found that the peptides present in the glycine HCl extracts of the immune precipitates could be reprecipitated completely by the addition of SDS to a final concentration of 0.1%. These insoluble peptides could be resolubilized in 0.1% SDS in TBS, pH 7.5. This extra step in the immunoprecipitation procedure eliminated heavy nonspecific radiolabeled bands exhibited on the autoradiograms. This procedure was routinely used to test sera in this study.

When extraction of the immune precipitate was carried out with 1% SDS instead of glycine HCl, a fourth band of 260 kD MW could be detected on reduced 5% SDS PAGE (Fig 1*B*). If the 1% SDS extract was boiled prior to electrophoresis, no 260-kD band could be detected. Instead, the lower-MW bands appeared to increase in intensity.

As shown in Figure 2*A*, two or three nonspecific bands (sometimes very intense) were precipitated by NHS. Figure 2*B* shows the autoradiographs of the labeled FS antigen precipitated by FS pa-

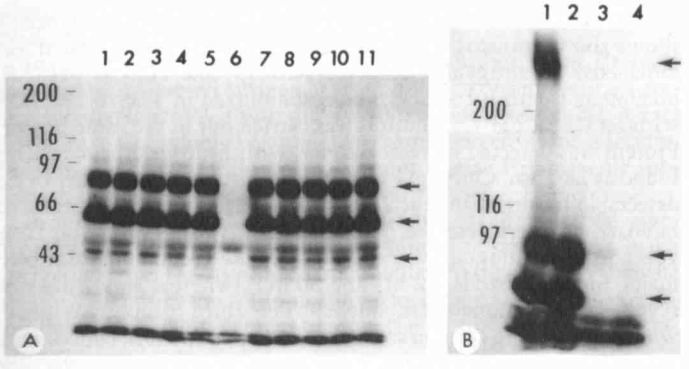

Figure 1. Autoradiographs of SDS 10% PAGE of immunoprecipitates of ^{125}I-labeled FS antigen(s). *A*, *Lanes 1–5* and *7–11* contain immunoprecipitates obtained by a panel of FS sera. The immunoprecipitates (Staphylococcus aureus cells [SaC]-immune complex) were extracted with glycine HCl, pH 2.8. *Lane 6* contains an immunoprecipitate obtained with normal human serum (NHS). Three bands *(arrows)* are specifically recognized by all FS sera tested (80-kD, 62-kD, and 45-kD). *B*, An FS patient's serum *(lanes 1, 2)* and NHS *(lanes 3, 4)* immunoprecipitate, extracted with 1% SDS and run unboiled *(lanes 1, 3)* and boiled *(lanes 2, 4)* on reduced 5% SDS gels. *Arrows,* positions of FS-specific bands. It appears that FS antigen extracted with 1% SDS and separated by SDS PAGE under reducing conditions migrate as a large MW heterotetramer (MW 260 kD), which is dissociated by either boiling or low pH into the 80-kD and 62-kD components.

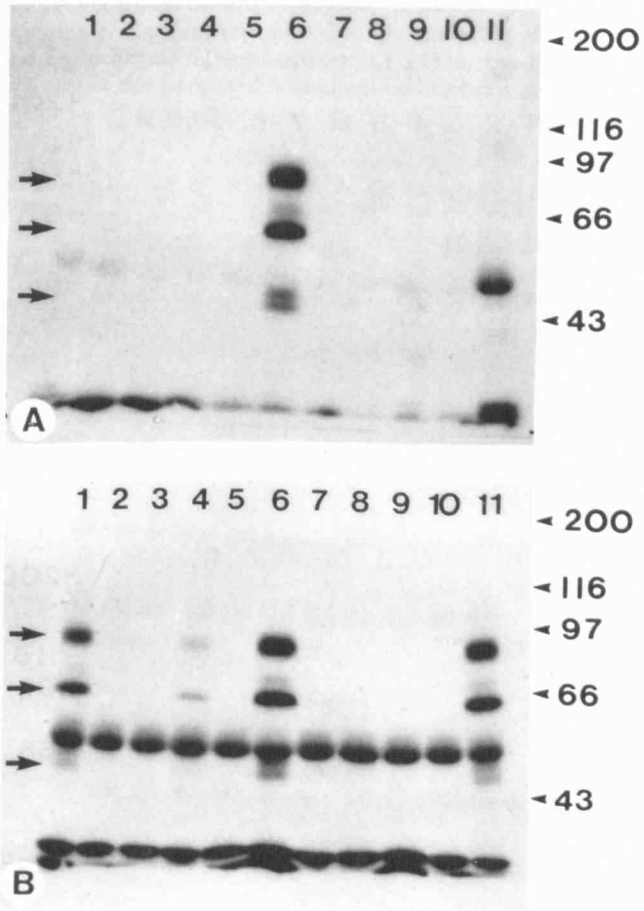

Figure 2. Autoradiographs of SDS 10% PAGE gels of immunoprecipitates of ^{125}I-labeled FS antigen(s). Immunoprecipitation carried out with *(A)* NHS *(lanes 1–5, 7–11), (B)* sera from FS patients in remission *(lanes 1–5, 7–11). Lane 6* in both *A* and *B* is FS serum for comparison. *Arrows,* positions of FS-specific bands. Whereas NHS did not precipitate any specific radiolabeled band, FS sera from patients in clinical remission exhibiting low titers of autoantibodies immunoprecipitate the FS antigen bands *(lanes 1, 4, 11). Panel B* of this figure also shows a heavy nonspecific radiolabeled band above the 45-kD FS antigen band. This nonspecific band was heavy in some radiolabeled epidermal extracts (see Figs 2*B* and 3*A,B*) or weak or absent in others (see Figs 1 and 2*A*).

tients in remission. Only FS sera with detectable autoantibodies (*lanes 1 and 11,* indirect IF titer of 1:160 and *lane 4,* titer of 1:40) precipitated the FS antigen bands; the rest of these sera (n:7) showed negative results.

Autoradiographs of PV sera and BP sera tested with the radiolabeled FS antigen are shown in Fig 3*A* and *B*, respectively. Five of 11 PV sera tested were capable of precipitating the three FS antigenic bands, whereas BP sera (n:11) showed negative results.

Figure 4 shows the results of the immunoprecipitation reaction using murine monoclonal antibodies against human IgG4 and IgG1,2,3 subclasses as second antibody instead of SaC. Two FS patients (GON and GO-160), having both IgG1 and IgG4 autoantibodies to FS antigen(s) by indirect IF, were used. In both cases the autoradiograms obtained wth the anti-IgG1,2,3 antibodies exhibited significantly decreased intensity in the 80-kD band and perhaps even complete absence of the 45-kD band.

Reaction of FS Antigen(s) with Anti-DG1 Sera Figure 5 shows the results of immunoprecipitation with rabbit anti-bovine DG1 and DG2 sera on the FS antigen extract. No bands (other than nonspecific bands) could be detected by these sera. By western immunoblotting (Fig 6) the anti-DG1 serum and FS serum recognize the same band, of approximately 165-kD MW, with 1% SDS epidermal extracts run on 7% SDS PAGE. However, whereas the anti-DG1 autoantibody activity could be adsorbed from the patient's serum with the human FS antigen(s), similar results could not be obtained by adsorption of the rabbit anti-bovine DG1 serum with the same FS antigen. As previously stated, the adsorbed FS serum showed no reactivity with human epidermal cryosections by indirect IF.

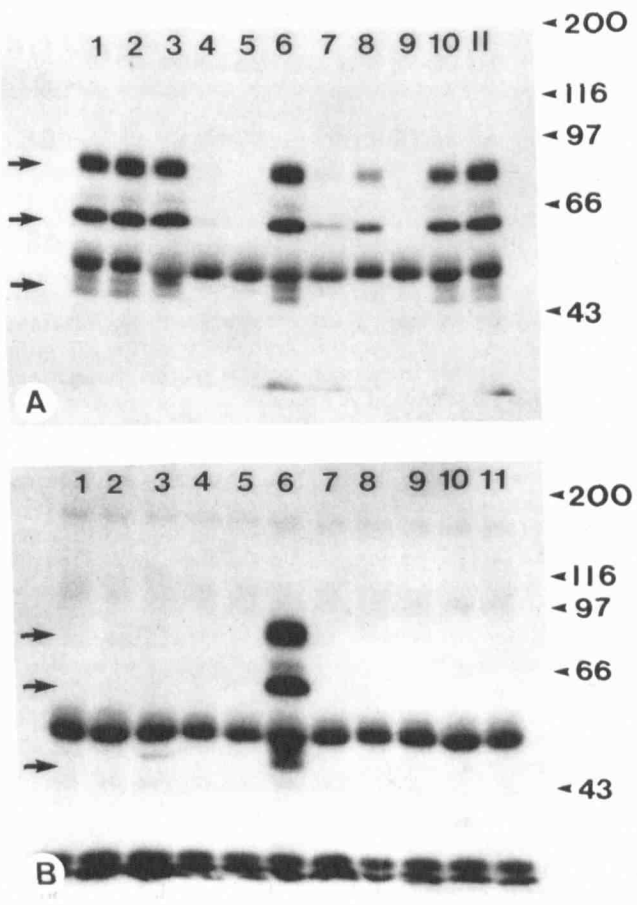

Figure 3. Autoradiographs of SDS PAGE on 10% gels of immunoprecipitates of ^{125}I-labeled FS antigen(s). Immunoprecipitation carried out with *(A)* sera from pemphigus vulgaris (PV) patients *(lanes 1–5, 7–11), (B)* sera from bullous pemphigoid (BP) patients *(lanes 1–5, 7–11). Lane 6* in both *A* and *B* is FS serum for comparison. *Arrows,* positions of FS-specific bands. Six PV sera precipitated the 80-kD and 62-kD FS antigens, whereas all BP sera showed negative results. *A* and *B* also show the nonspecific band above ovalbumin precipitated by all sera, as described in Fig 2*B*.

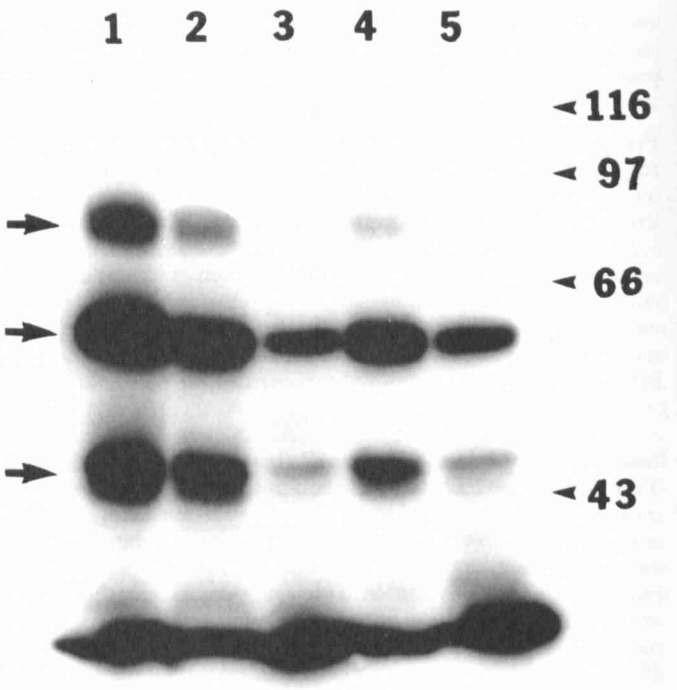

Figure 4. Autoradiographs of SDS 10% PAGE of immunoprecipitates of ^{125}I-labeled FS antigen(s) and IgG subclass autoantibodies. The radiolabeled FS antigenic fraction was immunoprecipitated with two FS patients' sera using agarose-conjugated anti-human IgG4 *(lanes 2, 4)* or anti-human IgG1,2,3 *(lanes 3, 5)* instead of SaC. *Lanes 2, 3* (Patient GON) and *lanes 4, 5* (patient GO-160). A control immunoprecipitate using FS serum (GON) and SPA *(lane 1)* is included for comparison. *Arrows,* positions of FS-specific bands. Whereas the 80-kD, 62-kD, and 45-kD FS antigens were immunoprecipitated by IgG4 autoantibodies, it appears that the 80-kD and 45-kD FS antigen(s) were not recognized by IgG1,2,3 autoantibodies.

Fractionation of FS Antigen(s) on Agarose Gel Figure 7 shows the chromatogram obtained with immunoprecipitated radiolabeled FS antigen(s) extracted with glycine HCl at pH 2.8, precipitated with 0.1% SDS, and resolubilized in TBS/0.1% SDS with no Ca^{++}, pH 7.5. Elution was carried out in the same buffer. Protein was detected by radioactivity, with little absorbance being found at 280 nm. Only IgG was present in sufficient quantity to be detectable by absorbance at 280 nm *(peak 1)*. Two major peaks of radioactivity were detected *(peaks 2 and 3)*. By SDS PAGE on 10% gels and autoradiography (Fig 7, *inset*), *peak 2* contained the two major antigenic bands (62 kD and 80 kD), whereas *peak 1* did not reveal any additional components. The third radioactive peak, which appeared to contain some absorbance at 280 nm, contained a low-MW component (<10 kD). These components, however, are non-specifically bound by all sera. The MW of the peak containing the FS antigen(s) was estimated to be 115–120 kD by comparison with standards.

DISCUSSION

We have reported recently that when viable human epidermis is trypsinized, a soluble FS antigen glycopeptide of 45 kD is released into the media [9]. Viable trypsin-dissociated keratinocytes no longer bind FS autoantibodies [14]. In a related study [8], we have shown that human epidermis separated from the dermis by raising the temperature to 56°C and sequentially extracting it in non-ionic detergents and 1.5 M KCl contained an insoluble pool of FS antigen

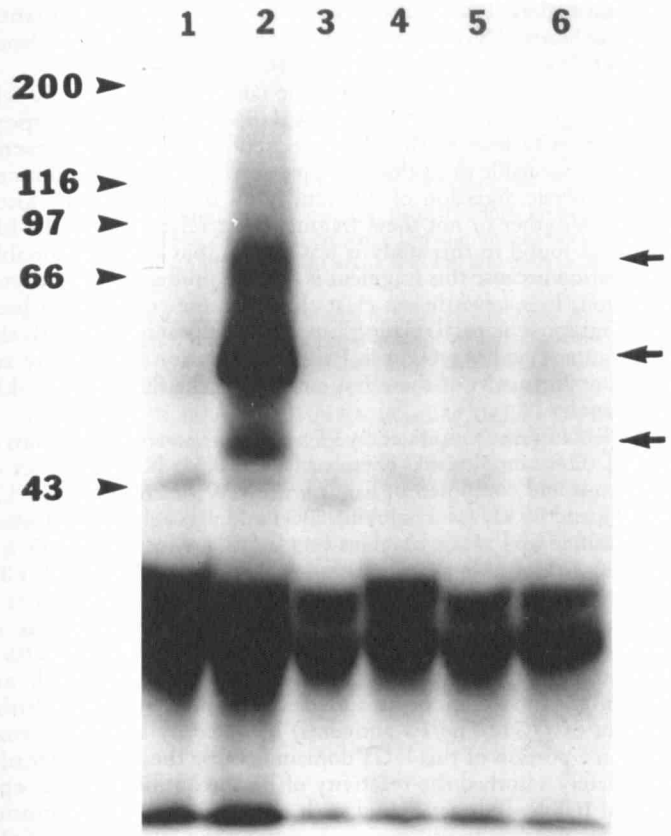

Figure 5. Autoradiographs of SDS PAGE on 10% gels of immunoprecipitates of ^{125}I-labeled FS antigen(s). Immunoprecipitation was carried out with NHS *(lane 1)*, FS serum *(lane 2)*, normal rabbit sera *(lanes 3, 5)*, rabbit anti-DG2 *(lane 4)* and rabbit anti-DG1 *(lane 6)*. SaC were used to precipitate immunoglobulins in each case. *Arrows*, positions of FS-specific bands. Only the FS serum (GON) immunoprecipitated the three FS antigen bands *(lane 2)*.

that remained bound to the envelope fraction after trypsinization. The FS antigen from the epidermal envelopes was released by proteolysis with papain and shown to be specifically bound by FS autoantibodies by immunoprecipitation techniques.

In the present paper, we report that sonication is an alternate method to release FS antigen(s) from human trypsin-resistant epidermal envelopes. Without the trypsinization step, sonication does not release any soluble FS antigen(s) from these envelope fractions. Moreover, trypsinization alone does not release FS antigenic substances from these insoluble pellets.

In agreement with the known insolubility of the epidermal envelope fraction [15], not even 1% SDS solubilizes any protein or FS antigen(s) from the trypsin-resistant pellets. This suggests that the physical shearing stress of sonication is an important factor in breaking down the cohesive forces binding the FS antigen(s) to the keratinocyte envelopes.

The amount of antigen released, though sufficient to adsorb FS autoantibody, could not be detected by absorbance at 280 nm after chromatography on agarose or by Coomassie blue staining on polyacrylamide gels. We estimated that 200 μg of protein-containing antigen adsorbed approximately 10–15 μg of antibody from 1 ml of 1:80 dilution of a high-titer FS serum (1:1280). Assuming equivalent labeling of the protein in this solution by ^{125}I, approximately 0.1% of the protein (0.2 μg) was precipitated by specific autoantibody. This low estimate is supported by the lack of optical density and absence of staining of this fraction on SDS PAGE. The remaining protein is apparently low in MW because it appears to be lost when ultrafiltered through membranes with 30-kD MW cutoffs.

When analyzed by immunoprecipitation, two major bands were obtained with MW of approximately 80 and 62 kD. A third band, which varies in intensity, but is usually weak, was also found at 45 kD. These results were obtained when immunoprecipitates were extracted at low pH with glycine HCl (pH 2.8). When extracted with 1% SDS, an additional labeled band was obtained at 260 kD. At present we do not know what the relationship is between these individual bands. Our speculation, based on observations of intensity and other properties, is that the 80-kD and 62-kD polypeptides are the major structural units. A heterodimer of these polypeptides would have a MW of approximately 140 kD, which approximates the MW of these FS antigen bands obtained by gel filtration (115–120 kD). The 45-kD polypeptide may be a breakdown product of one of these components, which appear to be joined noncovalently. The 260-kD moiety may be a polymer of the basic 140-kD unit. Of course, other explanations are possible and additional studies will be required to determine the correct relationships.

Antigenic activity of the sonicated extract is lost on boiling, repeated freezing and thawing, 50% saturated ammonium sulfate, and low pH (2.5). Lyophilization only partially inactivated it, whereas overnight incubation at 37°C, acetone (50%) precipitation, concentration by positive pressure using membranes with a 30-kD cutoff, and reduction and alkylation did not appear to affect it. These results suggest that the FS antigenic moieties released by sonication may be glycopeptides that are stable when maintained in TBS/Ca^{++}.

Immune precipitates redissolved in glycine HCl, pH 2.8, were found to precipitate in the presence of 0.1% SDS. This appears to be a property of the associated immunoglobulins because isolated FS

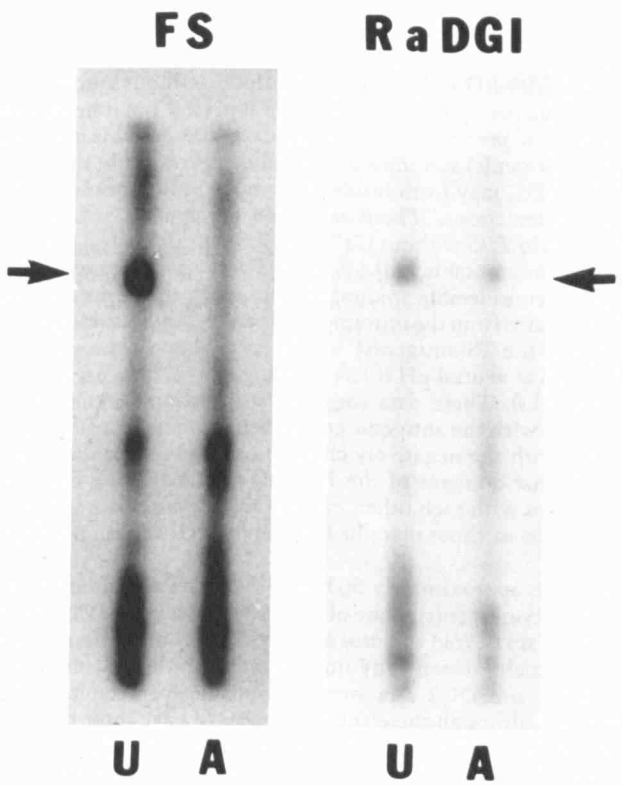

Figure 6. Autoradiographs of immunoblots of SDS PAGE of 1% SDS extracts of epidermis. Blots were incubated with FS serum diluted 1:160 or rabbit anti-DG1 (RaDG1) diluted 1:500 and ^{125}I-labeled Staphylococcus aureus protein A. The blots were probed with the same FS and rabbit anti-DG1 sera before *(U)* and after *(A)* adsorption with epidermal FS antigen(s) obtained by sonication. The adsorbed FS serum became unreactive with human epidermis by indirect immunofluorescence and no longer reacted with DG1 by immunoblotting *(FS panel, second lane)*. *Arrows*, position of DG1 recognized by unadsorbed FS serum and the rabbit anti-DG1 serum.

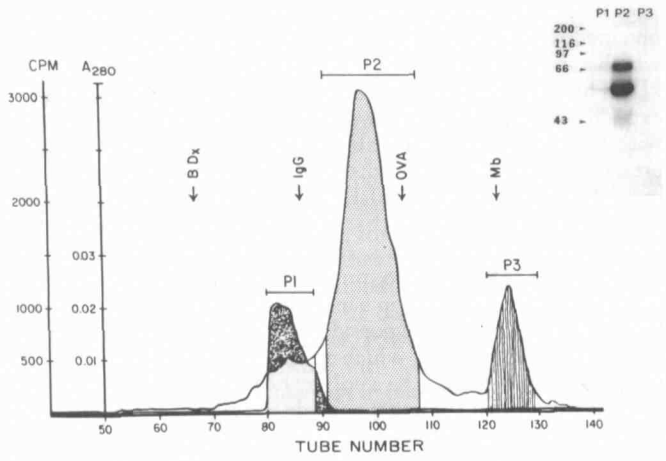

Figure 7. Bio Gel A 1.5M elution of ^{125}I-labeled FS antigen obtained by precipitation with FS serum (GON). The immune precipitates were redissolved in glycine HCl, pH 2.8, followed by reprecipitation with 0.1% SDS. The precipitate was then dissolved in TBS, pH 7.5, with 0.1% SDS without Ca^{++} and chromatographed in the same buffer. Absorbance was read at 280 nm and each tube counted in a gamma counter. *Inset* shows autoradiograph of the SDS PAGE (10% gel) of peaks 1, 2, and 3 (P1, P2, P3, respectively). Positions where standards eluted are also shown: blue dextran (BDx, MW $> 2 \times 10^6$), IgG (MW 160 kD), ovalbumin (Ova, MW 43 kD), and myoglobin (Mb, MW 17 kD). Peak 1 absorbed UV light at 280 nm but had low radioactivity. This peak contained IgG. Peak 2 contained the radiolabeled 80-kD and 62-kD FS antigen bands and showed no absorbance at 280 nm. Peak 3 contained low-MW radiolabeled peptides.

antigen(s) (80-kD and 62-kD bands) alone, without immunoglobulins, was not precipitated by 0.1% SDS at pH 2.8, but immunoglobulins alone are precipitated under these conditions. This may suggest that FS antigen(s) and immunoglobulin, at low pH in the presence of 0.1% SDS, may form insoluble complexes (nonimmune) due to charge interactions. These insoluble precipitates redissolved in 0.1% SDS in TBS without Ca^{++}, pH 7.5. At this neutral pH, immunoglobulins are stable in 0.1% SDS. These properties were used to remove a considerable amount of nonspecifically adsorbed radiolabeled proteins from the immunoprecipitates. It was also found, however, that the FS antigen(s) would precipitate in the presence of 0.1% SDS at neutral pH if left standing at 4°C for a period of time (2–3 weeks). These data suggest that a cationic moiety may be associated with the antigenic component, which is capable of complexing with the negatively charged dodecyl sulfate ion. It is also possible that domains of the FS antigen(s) may have homophilic interactions with each other, causing them to associate and precipitate, similar to those described for neural cell adhesion molecules [16].

Whereas approximately 50% of PV sera tested immunoprecipitated the FS antigen(s), none of the tested BP sera did. FS patients in remission are devoid of autoantibody activity and, therefore, they also are usually negative by immunoprecipitation. Rabbit anti-bovine DG1 and DG2 sera were also unable to precipitate the FS antigen(s), although these antisera appear to have some crossreactivity with human DG1 and DG2 by immunoblot analysis. In agreement with Stanley [17], our studies indicate that human DG1 is recognized by FS sera as well as by rabbit anti-bovine DG1 on immunoblotting. Furthermore, the FS antigen(s) obtained by sonication of insoluble epidermal pellets completely adsorbed the reactivity of FS serum with epidermal cryosections by indirect IF and with epidermal DG1 by immunoblotting, but did not completely remove reactivity of the rabbit anti-bovine DG1 serum with human epidermal DG1. It is possible that the sonicated epidermal extract contains all of the human DG1 epitopes recognized by FS autoantibodies but not all the epitopes recognized by the rabbit anti-bovine DG1 antibodies. Thus, whereas it is capable of adsorbing autoantibodies to human DG1, it is incapable of adsorbing all rabbit antibodies reactive with epitopes on bovine DG1.

The relationship of these antigenic components to the 50-kD papain fragment reported by Labib et al [8] or the 45-kD glycopeptide previously described by Martins et al [9] is unknown at present. It seems reasonable to assume that these are limit peptides produced by proteolytic digestion of different pools of epidermal FS antigen(s). Whether or not these fragments are related to the 45-kD fragment found in this study is not known, but it is a reasonable supposition because this fragment is also the product of proteolytic digestion. It is nevertheless clear that all these components bear some immunochemical relationship, as they appear to react with the same autoantibodies present in FS sera. Sonication may simply release the precursors of these fragments, i.e., the 80-kD or 62-kD components.

A PF complex precipitated by PF sera was reported by Korman et al [18]. This complex was obtained from a 0.5% NP-40 extract of epidermis and composed of bands with MW of 260 kD, 160 kD (DG1), and 85 kD (plakoglobin). DG1 and plakoglobin are linked by disulfide and other covalent bonds [18] to form the 260-kD protein. DG1 is thought to possess the antigen binding site for FS autoantibodies, which would suggest that the 50-kD of Labib et al [8], the 45-kD fragment of Martins et al [9], and possibly one or more of the FS antigen(s) reported in this paper are related to DG1. Our studies suggest strongly that FS autoantibodies, which are known to bind keratinocyte surfaces, recognize the extracellular domain of DG1. The FS antigen(s) obtained by sonication may contain a portion of this DG1 domain because these FS antigen(s) completely adsorbed the reactivity of FS autoantibodies with epidermal ICS by indirect IF and with epidermal DG1 by immunoblotting. Work is in progress to determine the relationship of the 80-kD, 62-kD, and 45-kD bands with plakoglobin and other desmosomal proteins as well.

The studies with individual subclasses of IgG suggest that there are differences in the ability of IgG1 and IgG4 to precipitate both the 80-kD and 45-kD polypeptides. IgG4 appears to react with all three components whereas IgG1 may not. Both subclasses appear to react with the 62-kD moiety. If this difference in specificity of the two subclasses is substantiated by additional studies, it may suggest that there are subtle differences in the diseases manifested by individual subclasses of IgG. Previous studies have implicated IgG4 as pathogenic by passive transfer studies [2], but the role of IgG1 is not clear.

The release of these FS antigenic fragments by sonication of the trypsin-resistant epidermal pellet represents a new method of obtaining antigenic components reactive with FS sera. At present we are attempting to generate larger amounts of these antigens by scaling up this procedure so that we will be able to chemically characterize them. This would allow us to understand the relationship between them as well as with those antigens reported by other investigators.

REFERENCES

1. Diaz LA, Sampaio SAP, Rivitti EA, Martins CR, Cunha PR, Lombardi C, Almeida FA, Martins-Castro R, Macca ML, Lavrado C, Filho GH, Borges PC, Chaul A, Minelli L, Empinotti JC, Friedman H, Campbell I, Labib RS, Anhalt GJ: Endemic pemphigus foliaceus (Fogo Selvagem): I. Clinical features and immunopathology. J Am Acad Dermatol 20:657–669, 1989
2. Rock B, Martins CR, Theofilopoulos AN, Balderas RS, Anhalt GJ, Labib RS, Futamura S, Rivitti EA, Diaz LA: The pathogenic effect of IgG4 autoantibodies in endemic pemphigus foliaceus (Fogo Selvagem). N Engl J Med 320:1463–1469, 1989
3. Koulu L, Kusumi A, Steinberg MS, Klaus-Kovtun V, Stanley JR: Human autoantibodies against a desmosomal core protein in pemphigus foliaceus. J Exp Med 160:1509–1518, 1984
4. Stanley JR, Klaus-Kovtun V, Sampaio SAP: Antigenic specificity of fogo selvagem autoantibodies is similar to North American pemphigus foliaceus and distinct from pemphigus vulgaris autoantibodies. J Invest Dermatol 87:197–201, 1986

5. Miller K, Mattey D, Measures H, Hopkins C, Garrod D: Localization of the protein and glycoprotein components of bovine nasal epithelial desmosomes by immunoelectron microscopy. EMBO J 6:885–889, 1987
6. Schmelz M, Duden R, Cowin P, Franke WW: A constitutive transmembrane glycoprotein of Mr 165000 (Desmoglein) in epidermal and non-epidermal desmosomes. II. Immunolocalization and microinjection studies. Eur J Cell Biol 42:184–199, 1986
7. Sotto MN, Shimizu SH, Costa JM, Brito T: South American pemphigus foliaceus: electron microscopy and immunoelectron localization of bound immunoglobulin in the skin and oral mucosa. Br J Dermatol 102:521–527, 1980
8. Labib RS, Camargo S, Futamura S, Martins CR, Rock B, Anhalt GJ, Diaz LA: Pemphigus foliaceus antigen: characterization of a keratinocyte envelope associated pool and preparation of a soluble immunoreactive fragment. J Invest Dermatol 93:272–279, 1989
9. Martins CR, Labib RS, Rivitti EA, Diaz LA: A soluble and immunoreactive fragment of pemphigus foliaceus antigen released by trypsinization of viable human epidermis. J Invest Dermatol 95:208–212, 1990
10. Parikh I, March S, Cuatrecasas P: Topics in the methodology of substitution reactions with agarose. Meth Enzymol 34:77–102, 1974
11. Matis WL, Anhalt GJ, Diaz LA, Rivitti EA, Martins CR, Berger RS: Calcium enhances the sensitivity of immunofluorescence for pemphigus antibodies. J Invest Dermatol 89:302–304, 1988
12. Labib RS, Anhalt GJ, Patel HP, Mutasim DF, Diaz LA: Molecular heterogeneity of the bullous pemphigoid antigens as detected by immunoblotting. J Immunol 136:1231–1235, 1986
13. Marier R, Jansen M, Andriole VT: A new method for measuring antibody using radiolabeled protein A in a solid-phase radioimmunoassay. J Immunol Meth 28:41–49, 1979
14. Diaz LA, Sampaio SAP, Rivitti EA, Macca ML, Roscoe JT, Takahashi Y, Labib RS, Patel HP, Mutasim DF, Dugan EM, Anhalt GJ: An autoantibody in pemphigus serum, specific for the 59 kD keratin, selectively binds the surface of keratinocytes: evidence for an extracellular keratin domain. J Invest Dermatol 89:287–295, 1987
15. Kubilus J, Kvedar J, Baden HP: Identification of new components of the cornified envelope of human and bovine epidermis. J Invest Dermatol 89:44–50, 1987
16. Hall AK, Rutishauser U: Visualization of neural cell adhesion molecule by electron microscopy. J Cell Biol 104:1579–1586, 1987
17. Stanley JR, Koulu L, Klaus-Kovtun V, Steinberg MS: A monoclonal antibody to the desmosomal glyoprotein desmoglein I binds the same polypeptide as human autoantibodies in pemphigus foliaceus. J Immunol 136:1227–1230, 1986
18. Korman NJ, Eyre RW, Klaus-Kovtum V, Stanley JR: Demonstration of an adhering-junction molecule (Plakoglobin) in the autoantigens of pemphigus foliaceus and pemphigus vulgaris. N Engl J Med 321:631–635, 1989

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